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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

**INTERNATIONAL APPL. NO.:** PCT/EP98/05770 :

**INTERNATIONAL FILING DATE:** -09-10-98- :

**APPLICANT:** THOMAS KIY :

**SERIAL NO:** : **ART UNIT:**

**FILED:** HERewith : **EXAMINER:**

**FOR:** "FERMENTATION PROCESS WITH CON-  
TINUOUS MASS CULTIVATION OF CILIATES  
(PROTOZOA) FOR PRODUCING BIOGENOUS  
VALUABLE SUBSTANCES" :

Assistant Commissioner of Patents  
Box PCT  
Washington, D.C. 20231

"Express Mail" No.: EK219465103

Date: MARCH 17, 2000

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-Carrie A. McPherson-  
(Typed or printed name of mailing paper or fee)

*Carrie A. McPherson*  
(Signature of person mailing paper)

**TRANSMITTAL OF APPLICATION PAPERS  
TO U.S. DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. §371  
(37 CFR 1.494 OR 1.495)**

This Transmittal Letter is based upon PTO Form 1390 (as revised in May, 1993).

The above-identified applicant(s) (jointly with their assignee) have filed an International Application under the P.C.T. and hereby submit(s) to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. §371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. §371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. §371[f]) at any time rather than delay.
4. ☒ A proper Demand for International Preliminary Examination (IPE) was made to the appropriate Authority (IPEA) within the time period required.
5. ☒ A copy of the International Application as filed (35 U.S.C. §371[c](2)) --
  - a. ☒ is transmitted herewith (required when not transmitted by International Bureau).
  - b. ☐ has been transmitted by the International Bureau. See WIPO Publication WO 99/15634.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A (verified) translation of the International Application into the English language is enclosed -with- Three (3) Sheets of Drawings.
7. ☐ Amendments to the (specification and) claims of the International Application under PCT Article 19 (35 U.S.C. 371[c](3))
  - a. ☐ are transmitted herewith (required if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
  - e. ☐ will be submitted with the appropriate surcharge.
8. ☐ A translation of the amendments to the claims (and/or the specification) under PCT Article 19 (35 U.S.C. §371[c](3)) is enclosed or will be submitted with the appropriate surcharge.

International Application No. PCT/EP98/05770

1997/F-237

9. ☒ An oath or declaration/power of attorney of the inventor(s) (35 U.S.C. §371[c][4]) is enclosed  
☒ and is attached to the translation of (or a copy of) the International Application.  
☐ and is attached to the substitute specification.  
☒ An associate power of attorney

10. ☐ A translation of at least the Annexes to the IPE Report under PCT Article 36 (35 U.S.C. §371[c][5]) is enclosed.

Items 11. to 16. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98 is enclosed.  
12. ☒ An Assignment is enclosed for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.  
13. ☒ A FIRST preliminary amendment is enclosed.  
A SECOND or SUBSEQUENT preliminary amendment is enclosed.  
14. ☐ A substitute specification (including claims, abstract, drawing) is enclosed.  
15. ☐ A change of power of attorney and/or address letter is enclosed.  
16. ☒ Other items of information:

- ☒ This application is being filed pursuant to 37 CFR 1.494(c) or 1.495(c), and any missing parts will be filed before expiration of--

☐ 22 months from the priority date under 37 CFR 1.494(c), or

☒ 32 months from the priority date under 37 CFR 1.495(c).

- ☐ The undersigned attorney is authorized by the International applicant and by the inventors to enter the National Phase pursuant to 37 CFR 1.494(c) or 1.495(c).

The following additional information relates to the International Application:

- ☒ Receiving Office: EPO
- ☒ IPEA (if filing under 37 CFR 1.495): EPO
- ☒ Priority Claim(s) (35 USC §§ 119, 365):  
German Appln. 197 41 489.3 filed September 19, 1997.
- ☒ A copy of the International Search Report is
  - ☐ enclosed.
  - ☒ attached to the copy of the International Application.
- ☒ A copy of the Receiving Office Request Form is enclosed.
- ☒ Form PCT/IB/306-1 sheet
- ☒ Form PCT/IB/308-2 sheets

The fee calculation is set forth on the next page of this Transmittal Letter.

## FEE CALCULATION SHEET

☒ A check in payment of the filing fee, calculated as follows, is attached (37 CFR 1.492).

Basic Fee..... \$ 840.00

Total Number of claims in  
excess of (20) times \$18..... -0-

Number of independent claims  
in excess of (3) times \$78..... -0-

Fee for multiple dependent  
claims \$260..... -0-

TOTAL FILING FEE... \$ 840.00

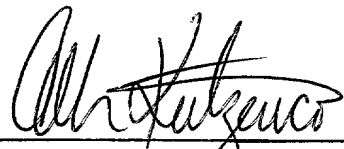
Kindly send us the official filing receipt.

The Commissioner is hereby authorized to charge any additional fees which may be required or to credit any overpayment to Deposit Account No. 03-2775. This is a "general authorization" under 37 CFR 1.25(b), except that no automatic debit of the issue upon allowance is authorized. An additional copy of this page is attached.

Respectfully submitted,

Date: -03/17/00-.

By

  
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ANK/cam (5822\*181)

Enclosures

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1997/F-237

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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THOMAS KIY

SERIAL NO:

FILED: HERewith

FOR: "FERMENTATION PROCESS WITH  
CONTINUOUS MASS CULTIVATION OF  
CILIAES (PROTOZOA) FOR PRODUCING  
BIOGENOUS VALUABLE SUBSTANCES".....  
Assistant Commissioner

for Patents

Washington, D.C. 20231

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Commissioner of Patents, Washington, D.C. 20231

-Carrie A. McPherson-

(Typed or printed name) of  
person mailing paper or fee)

*Carrie A. McPherson*  
(Signature of person  
mailing paper or fee)

PRELIMINARY AMENDMENT

Sir:

Prior to the determination of the filing fee and any action  
on the merits of the accompanying new patent application, kindly  
amend the application as follows:

In the Claims:

Claim 3, line 1, delete "or 2" ;

Claim 4, line 1, change "any of claims 1 to 3" to read --  
claim 1 -- ;

Claim 5, line 1, change "any of claims 1 to 4" to read --  
claim 1 -- ;

Claim 7, line 1, change "any of claims 1 to 6" to read --  
claim 1 -- ;

Claim 9, line 1, change "any of claims 1 to 8" to read --  
claim 1 -- ;

Claim 10, line 1, change "any of claims 1 to 9" to read --  
claim 1 -- ;

Claim 11, line 1, change "any of claims 1 to 10" to read  
-- claim 1 -- ;

Claim 12, line 1, change "any of claims 1 to 11" to read  
-- claim 1 -- ;

Claim 13, line 1, change "any of claims 1 to 12" to read  
-- claim 1 -- ;

Claim 14, line 1, change "any of claims 1 to 13" to read  
-- claim 1 -- .

R E M A R K S

Claims 3-5, 7 and 9-14 have been amended to refer to only  
one preceding claim. Each of the dependent claims, as amended,  
now depends on only one preceding claim. Therefore no  
additional fee is required for multiple dependency.


Prompt, favorable action is solicited.

Respectfully submitted,

CONNOLLY BOVE, LODGE & HUTZ LLP

Date: -03/17/00-.

By

  
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Fermentation process with continuous mass cultivation of ciliates (protozoa) for producing biogenous valuable substances

Description

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The invention relates to a fermentation process with continuous mass cultivation of ciliates (protozoa) for producing biogenous valuable substances, where the biomass containing the desired biogenous valuable substances is obtained by continuous (permanent) cell extraction.

10

To date, only initial attempts have been made in the biotechnological utilization of ciliates - a class of protozoa -, although a large number of metabolites of these organisms are of economical interest, for example lysosomal enzymes. As yet, only few biotechnological processes for obtaining biogenous valuable substances from ciliates have been described, - mainly for the ciliate Tetrahymena (Kiy & Tiedtke, 1991, Appl. Microbiol. Biotechnol., 35, 14; Kiy et al., 1996, Enzyme Microb. Technol., 18, 268; Kiy & Tiedtke, 1992, Appl. Microbiol. Biotechnol., 38, 141), - and these are exclusively processes for obtaining excreted cell products, i.e. those cell products which are given off by the ciliate cells into the culture medium. In this kind of process, the ciliates are cultivated in fermenters and the culture medium containing the excreted biogenous valuable substances is removed periodically, in more or less regular intervals, and exchanged for fresh medium. During the exchange of the medium, the ciliates are retained in the fermenter using certain methods - for example the use of membranes, cell immobilization and the like -, so that virtually no cell material is lost and the cell culture exists in principle permanently.

25

However, to obtain biogenous valuable substances which are attached to the cell it is necessary to harvest the total of the cells, the so-called biomass. To this end, in general - i.e. in the generally known fermentation processes with bacteria or fungi as producers of valuable substances - a so-called batch fermentation is carried out, where the fermenter is seeded and the cells are cultivated until the maximum biomass or product concentration has been reached. The biomass is then harvested. Such processes have already been described for various ciliates such as Paramecium, Colpoda and Tetrahymena (Proper & Garver, 1966, Biotechnol. Bioeng., 8, 287, Schönefeld et al., 1986, J. Protozool., 33, 222; Kiy & Tiedtke, 1992, Appl. Microbiol. Biotechnol. 37, 576).

35



However, batch fermentation processes have the fundamental disadvantage that, at intervals, cleaning, re-seeding of the fermenter and an intensive monitoring and looking after of the cell culture is required - in particular during the critical growth phase.

From the fermentation technique with bacteria or yeasts as producers of valuable substances, in addition to the batch fermentation process, the "continuous fermentation process" is also known. In this process, the cells are cultivated in the fermenter until they have reached a certain cell density and then permanently harvested by continuous cell extraction from the fermenter, while at the same time fresh culture medium is added to the same extent. The amount of cells extracted per time unit (the cell extract) is such that the cells which remain in the fermenter can easily recompensate the reduction of the cell density due to the harvest, by continuous cell divisions. In the range if a certain cell extraction rate or dilution rate "D", the cell density in the fermenter thus remains constant, although culture and accordingly the desired product are harvested continuously.

In principle, this continuous fermentation process is economically far superior to batch fermentation; however, its realization requires that the cultivated or bred organisms grow and multiply relatively quickly and uniformly, and that they are insensitive to the stirring and shear forces which are encountered in a continuous fermentation process.

Of ciliates, however, it is generally known that frequently they grow and multiply only very slowly, that they pass through different growth phases and that they react very sensitively to stirring and shear forces (Curds & Cockburn, 1971, Journal of General Microbiology 66, 95-109; Middler & Finn, 1966, Biotechnology and Bioengineering 8, 71-84). Attempted continuous mass cultivations of ciliates have indeed been described, but exclusively with the use of bacteria-containing culture media and with resulting maximum cell densities of a few ten thousand cells per ml, despite a cultivation of 10 days or more (Curds & Cockburn, supra.).

For use on an industrial scale, such cell densities are totally insufficient. In addition, the cultivation described by Curds & Cockburn is therefore also completely unsuitable for industrial use because it prescribes the use of a medium containing prey organisms, i.e. bacteria. In a bacteria-containing culture medium, the bacteria do, of course, also multiply permanently, the

extent depending on how many ciliates are present. The coexistence equilibrium of ciliate population and bacteria population is very labile, and even a slight intervention can cause substantial changes in both populations.

5

Furthermore, the experiments of Curds & Cockburn were carried out more than 25 years ago, and they have apparently confirmed the opinion of those skilled in the art that ciliates are unsuitable for a continuous fermentation process on an industrial scale.

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It is an object of the present invention to provide a process for continuous fermentation of ciliates with cell extraction, which avoids the abovementioned disadvantages and is very suitable, in particular, for industrial use.

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This object is achieved by a process of the type mentioned at the outset, where the ciliate cells are cultivated in a complex axenic medium.

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For the purpose of this invention, a complex medium is a nutrient medium in aqueous solution of natural products or extracts obtained therefrom for cultivating microorganisms.

25

For the purpose of this invention, axenic medium is a nutrient medium which is free of feed and prey organisms (so-called food organisms).

30

The process according to the invention is based on the surprising finding that a continuous fermentation process with cell extraction using complex axenic media can also be carried out successfully and in an economically highly rewarding manner using pure ciliate cultures. Continuous cell densities in an order of magnitude of 1 million cells per ml can be realized without any problems, in the case of Tetrahymena as early as from the third day after the beginning of the cultivation. Thus, the prejudice of those skilled in the art that ciliates are unsuitable for continuous mass cultivation with cell densities of several hundred thousand to millions of cells per ml using known fermenters and in the presence of the shear forces which are usually encountered, in axenic medium - i.e. without living feed or prey organisms, is overcome since:

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- they grow too slowly and not uniformly enough,

- they show only little resistance to stirring and shear forces and are very easily and rapidly damaged and/or destroyed by such forces, and
- in the cultivation attempts that have been carried out so far, in spite of a prey-organism-containing media being used, and thus a substantially natural diet, only relatively very low maximum cell densities have been reached.

Using the process according to the invention, it is possible for the first time to employ ciliates for the industrial production of biogenous valuable substances attached to the cell, and thus to obtain, on an economically important scale, in particular those valuable substances which are only known from ciliates, such as, for example, taurolipids and tetrahymanol, or those which are produced extensively specifically by ciliates, such as, for example, gamma-linolenic acid, docosahexaenoic acid, eicosapentaenoic acid, octatetraenoic acid and arachidonic acid.

Since the ciliates are kept as a pure culture - i.e. free from other living organisms -, important disturbing factors are avoided a priori, and even the technical expense is limited to a minimum: fermenters for regrowing the prey organisms, for example, are completely redundant.

The products which can be obtained from the extracted biomass include peptides and proteins, especially enzymes (for example  $\beta$ -hexosaminidase, L-asparaginase, diisopropylfluorophosphatase, glucosidase, fucosidase, phosphatase, nuclease or cathepsin.L), fatty acids and lipids (for example gamma-linolenic acid, docosahexaenoic acid, eicosapentaenoic acid, octatetraenoic acid, arachidonic acid, phosphonolipids, taurolipids or tetrahymanol), polysaccharides, nucleic acids, secondary metabolites, polymers, etc.

It is also possible for the biomass as such to be the product.

The group of the ciliates which can be cultivated by the process described includes all taxonomic ciliate sub-groups which can be cultivated in principle in conventional standing and/or shaking cultures or batch fermentations on axenic nutrient media or nutrient media which comprise, as nutrient, the killed biomass of a feed organism. These are, in particular, the ciliate sub-classes Holotricha, Peritricha, Spirotricha and Suctorina, and very particularly the orders Tetrahymena, Paramecium, Colpoda, Glaucoma, Parauronema, Engelmanniella, Styronichia, Euplotes and

Colpidium (classification according to K. Hausmann: Protozoologie, Thieme Verlag, 1985). Furthermore, the invention is not limited to wild strains but includes mutants and recombinant strains.

- 5 In a preferred embodiment of the process according to the invention, the fermentation is carried out in a stirred or bubble column or airlift fermenter.

During the fermentation, the pH can be regulated, preferably to a value in the range from pH 4 to pH 9.

- 10 Depending on the ciliate species, the fermentation temperature is between 15 and 40°C.

- As carbon source, preferably at least one of the substances listed below is used, i.e.: glucose, fructose, xylose, sucrose, maltose, starch, fucose,  
15 glucosamine, lactose, molasses, dextran, fatty acids (for example oleic acid), soya oil, sunflower oil, glycerol, glutamic acid, mannitol, skim-milk powder or acetate.

- The concentration of the carbon source should be between 0.2 and 20%  
20 by weight, based on the culture medium.

- As nitrogen source, preferably at least one of the substances listed below is used, i.e.: peptones, yeast extract, malt extract, meat extract, skim-milk powder, casamino acid, corn steep liquor, organic nitrogen sources, such  
25 as Na-glutamate and urea, inorganic nitrogen sources, such as ammonium acetate, ammonium sulfate, ammonium chloride or ammonium nitrate.

The concentration of the nitrogen source should be between 0.1 and 10% by weight, based on the culture medium.

- 30 In a variant of the process according to the invention, at least one phosphate source, for example potassium phosphate or potassium dihydrogen phosphate, is added to the culture medium. Alternatively or cumulatively, it is also possible to add ammonium sulfate, sodium sulfate, magnesium, iron, copper, calcium, vitamins, trace elements and growth  
35 factors, to further optimize the growth and multiplication rate of the ciliate culture in question.

The continuously harvested biomass is preferably separated off from the culture medium using centrifugation, tangential filtration, microfiltration,

sedimentation, flotation or separators. However, other methods are also feasible.

In a preferred embodiment of the process according to the invention, the  
 5 cell extraction rate or dilution rate  $D$  (= volume exchanged per day/operating volume of the fermenter) is in the range from 0.1 to 12 (= 1/10 to 12/1), depending on the growth rate of the ciliate strain.  
 Below, the invention is illustrated in more detail using working examples.

#### 10 Example 1: Continuous fermentation of *Tetrahymena pyriformis*

*Tetrahymena pyriformis* was cultivated in a 2 l fermenter of the Biostat MD type (Braun Biotech, Melsungen), under the following conditions:

15 Medium:

Water with additives of

- 0.5% by weight of Proteose Peptone
- 0.1% by weight of yeast extract
- 3% by weight of liquid starch sugar
- 20 - 1 ml/l of iron trace

Fermentation conditions:

- Temperature: 30°C
- Oxygen saturation: 20%
- 25 - pH regulation: pH 7
- Start ( $t_0$  h): inoculum 50,000 cells/ml; cultivation by the batch process type
- Beginning of the continuous fermentation:  $t_{51 \text{ h}}$  with  $D = 1$ ;
- Continuation of the continuous fermentation: from  $t_{78 \text{ h}}$  with  $D = 1.5$ ;
- 30 from  $t_{98 \text{ h}}$  with  $D = 2.4$

At the beginning of the cultivation, the medium was inoculated with about 50,000 cells, and this starter culture was cultivated by a batch process until the cell population was at the end of the multiplication phase and just  
 35 before entering the stationary phase. At this time - in the present example 51 hours after inoculation ( $t_{51 \text{ h}}$ ) -, the fermentation was changed to continuous fermentation, i.e. from then on cell-containing medium was continuously withdrawn, and the corresponding amount of cell-free medium was added. At the beginning of the continuous fermentation, the cell

extraction or dilution rate (= amount by volume of medium exchange per day per operating volume of the fermenter) was  $D = 1$ , i.e. per day, the entire content of the fermenter (2 l) was exchanged once, and 2 l of ciliate-containing medium were obtained. This medium contained about 1 million cells per ml.

27 hours after the beginning of the continuous fermentation (= 78 hours after the inoculation =  $t_{78 \text{ h}}$ ), the cell extraction rate or dilution rate was increased to  $D = 1.5$ , i.e. from this point of time onwards, about 3 l of ciliate-containing medium were obtained per day. The cell density was virtually unchanged at about 1 million cells per ml. After a further 20 hours (= 98 hours after the inoculation =  $t_{98 \text{ h}}$ ), the cell extraction rate or dilution rate was increased once more, to  $D = 2.4$ , i.e. about 5 l of ciliate-containing medium were obtained per day, the cell density being, as before, virtually unchanged at about 1 million cells/ml.

The results of this fermentation process are shown diagrammatically in Fig. 1. From the plot shown therein, it is evident that, although cells were continuously extracted, there was no dilution, but a permanent and continuous multiplication of the ciliates. In other words: even at a relatively large cell extraction ( $D = 2.4$ ), the culture was always in a dynamic equilibrium between cell extraction and cell multiplication.

#### Example 2: Continuous fermentation of *Tetrahymena thermophila*

*Tetrahymena thermophila* was cultivated in a 2 l fermenter of the Biostat MD type (Braun Biotech, Melsungen), under the following conditions:

##### Medium:

Water with additives of

- 5 g/l of Proteose Peptone
- 1 g/l of yeast extract
- 1 ml/l of iron trace
- 1% by weight of glucose in the form of liquid starch sugar

##### Fermentation conditions:

- Temperature: 30°C
- Oxygen saturation: 20%

- pH regulation: pH 7
- Start ( $t_0$  h): inoculum 50,000 cells/ml; cultivation by the batch process type
- Beginning of the continuous fermentation:  $t_{45}$  h with  $D = 1.2$ ;
- 5 - Continuation of the continuous fermentation: from  $t_{178}$  h with  $D = 2.4$ ;  
from  $t_{198}$  h with  $D = 3$

The process was carried out in principle as described under Example 1.

- 10 In Fig. 2, the growth or multiplication behavior of the ciliate population under the fermentation conditions mentioned is shown diagrammatically. From the plot shown, it is evident that the increase of the cell extraction or dilution rate from  $D = 1.2$  to  $D = 2.4$  (duplication) resulted in a reduction of the cell density from about 1 million cells/ml to about 500,000 cells/ml
- 15 (halving), but that this cell density then remained relatively constant and did not decrease any further, even when the cell extraction or dilution rate was increased further from  $D = 2.4$  to  $D = 3$ .

### Example 3: Continuous fermentation of *Tetrahymena thermophila*

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Medium:

Water with additives of

- 20 g/l of skim-milk powder
- 10 g/l of glucose
- 25 - 5 g/l of yeast extract
- 1 ml/l of iron trace

Fermentation conditions:

- Temperature: 30°C
- 30 - Oxygen saturation: 20%
- Stirrer: as 2. Cascade for oxygen regulation
- pH regulation: pH 7
- Start ( $t_0$  h): inoculum 50,000 cells/ml; cultivation by the batch process type
- 35 - Beginning of the continuous fermentation:  $t_{20}$  h with  $D = 1.125$ ;
- Continuation of the continuous fermentation
  - from  $t_{68}$  h with  $D = 1.9$ ;
  - from  $t_{139}$  h with  $D = 4.14$ ;

The process was in principle carried out as described under Example 1.

- 5 In Fig. 3, the growth or multiplication behavior of the ciliate population under the fermentation conditions mentioned above is shown diagrammatically. The plot shown indicates that, at the beginning of the continuous fermentation, the cell density decreased from initially about 1 million cells per ml to about 600,000 cells per ml. However, in spite of the  
10 cell extraction or dilution rate being increased, the cell population recovered within 1.5 days (about 36 hours) and, after about 4 days (90 hours) after the beginning of the continuous fermentation, had once more reached its initial density of 1 million cells per ml. Even when the cell extraction or dilution rate was increased further to  $D = 4.1$  and finally to  
15  $D = 4.9$ , the actual value was never again beneath this value.

The lower plot in Fig. 3 shows the results of determinations of the dry weight of the cells (in g per l) during the duration of the cultivation. The plot, which is essentially parallel to the curve for cell multiplication, shows  
20 that cell multiplication does not take place at the expense of the cell size or the cell volume of the individual ciliate cells, but that more biomass is indeed produced.

#### Example 4: Continuous fermentation of *Colpidium campylum*

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*Colpidium campylum* was cultivated in a 2 l fermenter of the Biostat MD type (Braun Biotech. Melsungen) under the following conditions:

Medium:

- 30 Water with additions of
- 20 g/l skim-milk powder
  - 10 g/l of glucose
  - 5 g/l of yeast extract
  - 1 ml/l of iron trace

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Fermentation conditions:

- Temperature: 25°C
- Oxygen saturation: 20%
- Stirrer: 108 rpm



- pH regulation: pH 7
- Start ( $t_0$  h): inoculum 50,000 cells/ml; cultivation by the batch process type
- Beginning of the continuous fermentation:  $t_{114.75}$  h with  $D = 0.665$ ;
- 5 - Continuation of the continuous fermentation
  - from  $t_{140}$  h with  $D = 0.632$
  - from  $t_{159}$  h with  $D = 0.462$

The process was carried out in principle as described under Example 1.

## Claims

1. A fermentation process with continuous mass cultivation of ciliates (protozoa) for producing biogenous valuable substances, where the biomass containing the biogenous valuable substances is obtained by continuous cell extraction, which comprises cultivating the ciliate cells in a complex axenic medium.
2. The fermentation process as claimed in claim 1, wherein the ciliates belong to one of the taxonomic groups Holotricha, Peritricha, Spirotricha and Suctoria, in particular to the orders Tetrahymena, Paramecium, Colpoda, Glaucoma, Parauronema, Engelmanniella, Stylonichia, Euplotes and Colpidium, which include, in addition to the wild strains, also mutants and/or recombinants of these strains.
3. The fermentation process as claimed in claim 1 or 2, wherein the fermentation is carried out in a stirred or bubble column or airlift fermenter.
4. The fermentation process as claimed in any of claims 1 to 3, wherein the fermentation is carried out at a pH in the range from pH 4 to pH 9 and/or a fermentation temperature in the range from about 15 to about 40°C.
5. The fermentation process as claimed in any of claims 1 to 4, wherein the medium contains a carbon source which comprises one or more substances from the group consisting of: glucose, fructose, xylose, sucrose, maltose, starch, fucose, glucosamine, lactose, molasses, dextran, fatty acids (for example oleic acid), soya oil, sunflower oil, glycerol, glutamic acid, mannitol, skim-milk powder and acetate.
6. The fermentation process as claimed in claim 5, wherein the concentration of the carbon source has a value in the range from about 0.2 to about 20% by weight, based on the culture medium.
7. The fermentation process as claimed in any of claims 1 to 6, wherein the medium contains a nitrogen source which comprises

one or more substances from the group consisting of: peptones, yeast extract, malt extract, meat extract, skim-milk powder, casamino acid, corn steep liquor, Na-glutamate, urea, ammonium acetate, ammonium sulfate, ammonium chloride and ammonium nitrate.

8. The fermentation process as claimed in claim 7, wherein the concentration of the nitrogen source has a value in the range from about 0.1 to about 10% by weight, based on the culture medium.

9. The fermentation process as claimed in any of claims 1 to 8, wherein the medium contains at least one phosphate source, preferably potassium phosphate and/or potassium dihydrogen phosphate.

10. The fermentation process as claimed in any of claims 1 to 9, wherein the medium contains one or more of the following substances: ammonium sulfate, sodium sulfate, magnesium, iron, copper, calcium, vitamins, trace elements.

11. The fermentation process as claimed in any of claims 1 to 10, wherein the medium contains killed biomass of feed organisms of the ciliates.

12. The fermentation process as claimed in any of claims 1 to 11, wherein the cells contained in the cell extract (= harvested biomass) are separated off from the culture medium by centrifugation and/or tangential filtration and/or microfiltration and/or sedimentation and/or flotation.

13. The fermentation process as claimed in any of claims 1 to 12, wherein the cell extraction rate or dilution rate (= the volume that is exchanged per day/the operating volume of the fermenter) has a value in the range from 0.1 to 12.

14. The fermentation process as claimed in any of claims 1 to 13, wherein the biogenous valuable substances are one or more substance(s) from the group consisting of: peptides and proteins, in particular enzymes, fatty acids and lipids, polysaccharides, nucleic

acids, secondary metabolites and polymers, or else the biomass itself is a valuable substance (for example animal feed).

## 97/F 237 WO

### Abstract

In the fermentation process according to the invention with continuous mass cultivation of ciliates (protozoa), the ciliate cells are cultivated in a complex axenic medium - free from living feed or prey organisms - and the biomass containing the desired biogenous valuable substances is obtained by continuous (permanent) cell extraction.

Figure 1

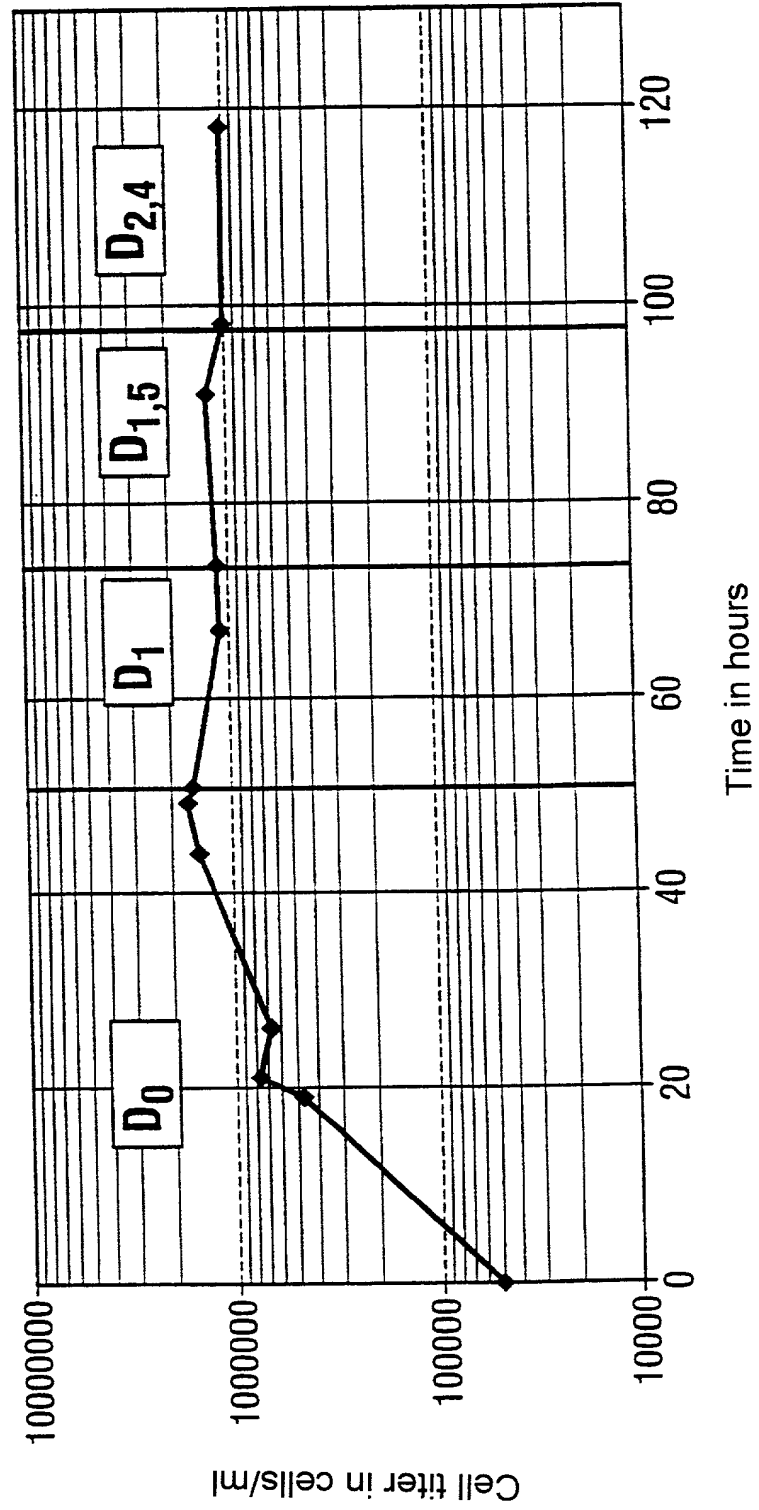


Figure 2

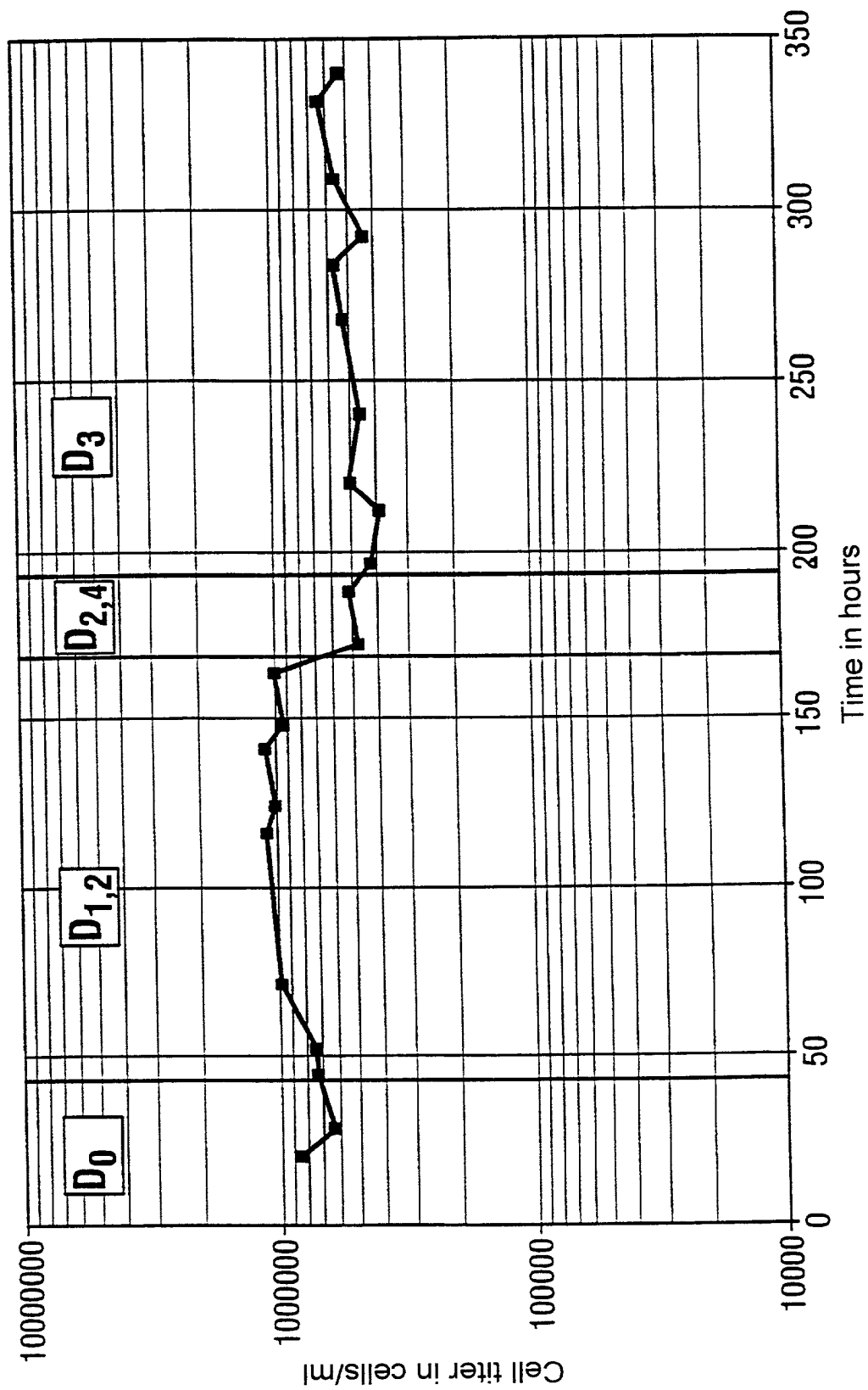
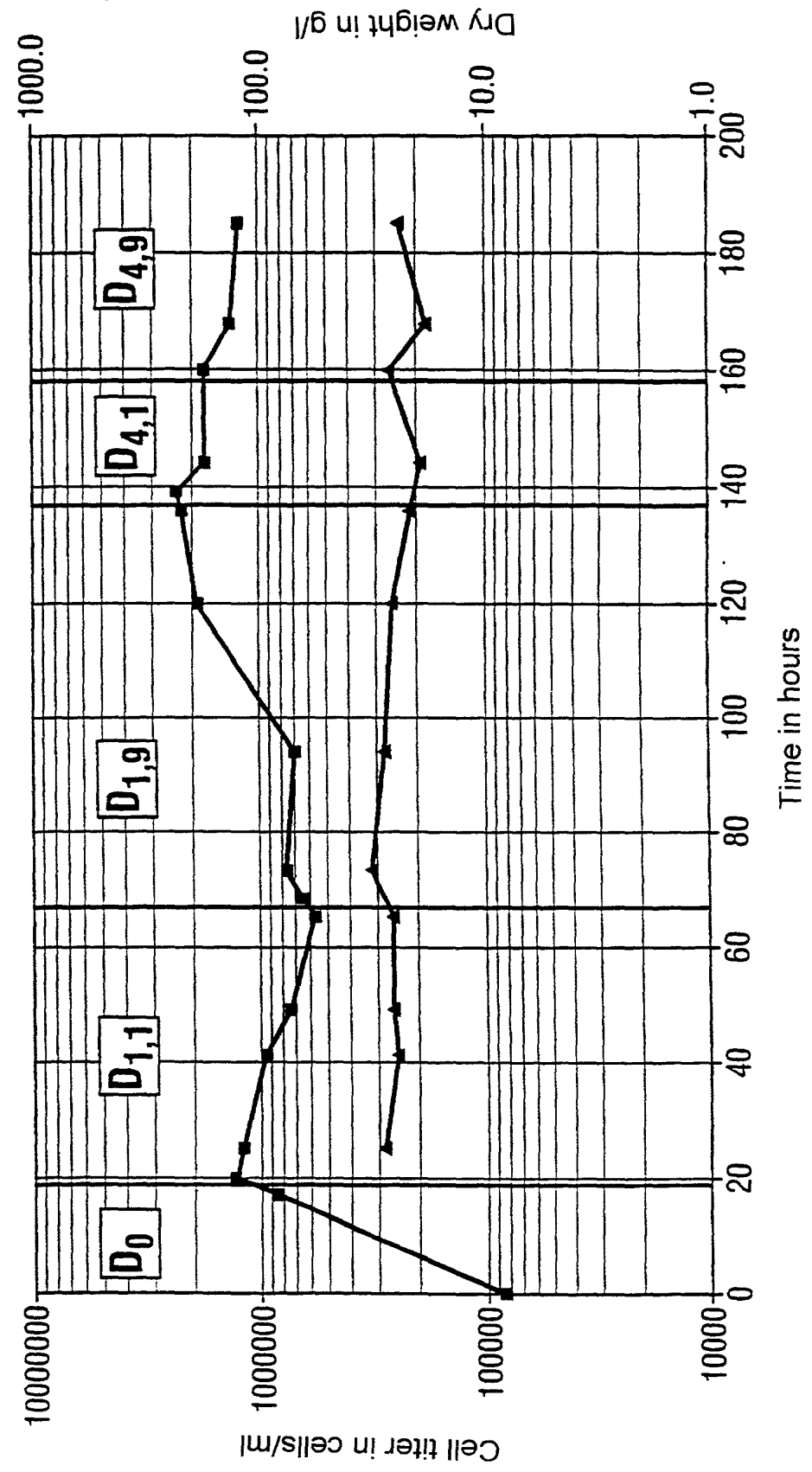


Figure 3





**COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY**

As below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below, I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Fermentation process with continuous mass cultivation of ciliates (protozoa) for producing biogenous valuable substances

the specification of which

■ is attached hereto

■ was filed on September 09, 1998 as International Patent Application PCT/EP98/05770 and including all the amendments through the date hereof.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

**Prior Foreign Application(s) for which Priority is Claimed:**

Federal Republic of Germany, 19741489.3 of September 19, 1997

And I hereby appoint

Albert F. Bower, Reg.No. 15,449; Rudolf E. Hutz, Reg.No. 22,397; Harold Pezzner, Reg.No. 22,112; John D. Fairchild, Reg.No. 19,756; Richard M. Beck, Reg.No. 22,580; Paul E. Crawford, Reg.No. 24,397; Thomas M Meshbesh, Reg.No. 25,083; Robert G. McMorrow, Jr., Reg.No. 30,962; Patricia Smink Rogowski, Reg.No. 33,791; Ashley I. Pezzner, Reg.No. 35,646; William E. McShane, Reg. 32,707; James T. Moore, Reg. No. 35,619

all of Connolly, Bove, Lodge and Hutz, Market St., Wilmington, Delaware 19899-2007, my attorneys with full power of substitution, to prosecute this application, and transact all business in the Patent and Trademark Office connected therewith and I hereby request that all correspondence in this application be directed to

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12

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

INVENTOR(S) / Residence

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Date: 31.01.00

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